PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael BUSCHLE *et al.*	Group Art Unit: 1648
Serial No.: 10/564,429	Examiner: Z. Lucas
Filed: April 24, 2006	Atty. Dkt. No.: SONN:084US
For: HCV VACCINES	Confirmation No.: 7323

DECLARATION OF CHRISTOPH KLADE

I, Christoph Klade, declare as follows:

1.I am an Austrian citizen residing at Gröhrmühlgasse 1B/17, 2700 Wiener Neustadt, Austria.

2.I am a co-inventor of the above-referenced patent application. I am currently a Vice President for Technology Development and Clinical Immunology at Intercell AG. I have extensive experience in the field of immunology, and more particularly, with the development of vaccines for viral diseases such as hepatitis C virus ("HCV") infections. A copy of my *curriculum vitae* is attached as Appendix A.

3.I have reviewed the Office Action of 6 November 2007, the specification, and the pending claims related to the above-referenced U.S. patent application.

4.I understand that the Examiner assigned to this application contends that claims 34, 35, 39, 40, 48, 60-62, 64, 65, 72, and 73 are not enabled. Although the Examiner acknowledges that the disclosed compositions are immunogenic, the Examiner argues that "the application has not

provided sufficient information to enable those in the art to treat and [*sic*] HCV infection with the claimed compositions." Office Action, p. 6.

5.I have attached as Appendix B the publication Firbas *et al.*, "Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: A randomized, placebo controlled trial for dose optimization in 128 healthy subjects," *Vaccine*, 24:4343-4353 (2006). This publication describes a clinical trial of an HCV vaccine called IC41. IC41 contains the following HCV peptides: Ipep 83, Ipep 84, Ipep 87, Ipep 89, and Ipep 1426. These peptides are recited in the current claims of the above-referenced patent application and correspond to SEQ ID NOs: 72, 60, 19, 17, and 63, respectively. In addition to the HCV epitopes, the IC41 vaccine also contains poly-L-arginine as an adjuvant. The IC41 vaccine was prepared in a similar manner to the compositions described in the present application. *See* Firbas *et al.* at 4344, col. 2; Application Serial No. 10/564,429, Examples I and V.

6.The IC41 HCV vaccine was analyzed to determine its overall safety and immunogenicity in 128 HLA A2 positive healthy humans. Firbas *et al.*, at 4344, col. 2. The study design included a single (subject) blind, randomized, controlled parallel group study in which three control groups and seven treatment groups were used. *Id.* at Table 1. The subjects received four vaccinations subcutaneously (one every 4 weeks for three months on Days 1, 29, 57, and 85). *Id.* at 4344, col. 2, to 4345, col. 1.

7.Immunization with IC41 HCV vaccine was generally safe and well tolerated. *Id.* at 4347-4348. In addition, poly-L-arginine increased the number of subjects who mounted an IFN-γ secreting T cell response, providing proof of concept for this adjuvant in humans. *Id.* at 4351, col. 2.

8.Interferon-gamma ELIspot assays, T cell Proliferation assays, and HLA tetramer-binding assays were carried out with cryopreserved PBMCs to assess the IC41 HCV vaccine immunogenicity. *Id.* at 4345, col. 2. As explained in Firbas *et al.*, "[t]hese types of assays allow reliable measurements of epitope-specific T cell responses induced by the therapeutic HCV vaccine IC41." *Id.* Moreover, "[t]he vaccine-induced T cell immune responses serve as surrogate parameters of efficacy." *Id.*

9.The efficacy results of the IC41 HCV vaccine were positive. *Id.* at 4348-4350. Table 3 on page 4348 of Firbas *et al.* summarizes the efficacy data. Figure 1 shows the time course of HCV peptide specific T cell proliferation in response to IC41 vaccination in a sub-analysis pooling data from all 61 proliferation responders. *Id.* a 4348. The data in Figure 1 confirms that HCV peptide specific T cell proliferation increased with the number of vaccinations and peaked after the last vaccination. *Id.* at 4349, column 1.

10.In summary, at least three conclusions can be drawn from the IC41 HCV vaccine safety and immunogenicity assays described in Firbas *et al.* First, the IC41 HCV vaccine was generally safe and well tolerated. *Id.* at 4347-4348. Second, the IC41 HCV vaccine/poly-L-arginine combination provokes a T-cell immune response in humans, and such a response serves as a surrogate parameter of efficacy. *Id.* at 4345, col. 2. Third, the data confirms the existence of a synergistic effect with co-administration of the IC41 HCV vaccine and poly-L-arginine enhances the induction of functional IFN-γ secreting T cells in humans. *Id.* at 4350, col. 2.

11.I declare that all statements made of my knowledge are true and all statements made on the information are believed to be true; and, further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereupon.

Date: Vienna Apr 3/2008

Cristoph Klade, Ph.D.

APPENDIX A
(*Curriculum Vitae*)

Curriculum Vitae

Name:		Christoph Stefan Klade
Date of Birth:		13 February 1969
Place of Birth:		Vienna, Austria
Nationality:		Austrian
Marital Status:		married to Manuela since 1997, daughter Lisa-Marie born February 4, 1998 son Lukas born October 19, 2002
Education:	1980-1987:	Bundesgymnasium Babenbergerring 2700 Wr. Neustadt, Austria
	May '87:	Awarded "Matura" (GCE A-level)
	1987-1988:	Army Service (compulsory)
	1988-1994:	Biochemistry, University of Vienna, Austria
	1994-1995:	Diploma Thesis "Identification and Characterization of Interferon/Immunoglobulin fusion proteins"
	Jun.'95:	Awarded "Magister rerum naturalium" degree (M.S.)
	1995-1998:	Ph.D. Thesis "Identification of tumor-associated antigens in renal cell carcinoma"
	Nov.'98:	Awarded Ph.D. ("Doctor rerum naturalium")
	1998 to date:	continuous education in bio-medical sciences including immunology (Berkeley, Stanford), Good Laboratory Practices, Radiation Safety and others; continuous education regarding Project Management, Leadership, Presentation and Communication skills
Working Experience:		
	1987-1994:	Programming and development of databases Rohoelaufsuchungs GesmbH (RAG) Schwarzenbergplatz 16, 1015 Vienna
	1994-1999:	Boehringer Ingelheim Austria GmbH Research & Development
	1999 to date:	InterCell AG, Vienna, Austria
	2000/2001:	Group Leader Proteomics Antigen Discovery
	2001/2002:	Head of T-cell epitope Identification Program
	since 2002:	Head of Clinical Immunology and T-cell epitope Identification
	since 2004:	Project Leader viral T cell vaccines
	since 2006:	Vice President Technical Development & Clinical Immunology Member of Senior Management Decision Bodies (Research & Development Committee, Clinical Development Council)
	since 2007:	leading co-development of the Hep C therapeutic vaccination program of Intercell and Novartis.
Miscellaneous:		Excellent English (TOEFL score 633 or 96%) Excellent presentation and communication skills Problem/decision analysis (Kepner-Tregoe) Certified Radiation Safety Officer

APPENDIX B

(Firbas *et al.*, *Vaccine*, 24:4343-4353 (2006))

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Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: A randomized, placebo controlled trial for dose optimization in 128 healthy subjects☆

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Abstract

As interferon/ribavirin-based standard therapy is curative in only about half of HCV patients, there remains an important need for alternatives including vaccines. The novel peptide vaccine IC41 consists of five synthetic peptides harboring HCV T cell epitopes and poly-L-arginine as synthetic adjuvant. In this randomized, placebo-controlled trial, 128 HLA-A2 positive healthy volunteers received four s.c. vaccinations of seven different doses IC41, HCV peptides alone, poly-L-arginine alone or saline solution, every 4 weeks. IC41 was safe and well tolerated. Mild to moderate local reactions were transient. Immunogenicity was assessed using T cell epitope specific [^{3}H]-thymidine proliferation, IFN-gamma ELIspot and HLA-tetramer assays. IC41 induced responses in all dose groups. Higher responder rates were recorded in higher dose groups and increasing number of vaccinations were associated with higher responder rates and more robust responses. Poly-L-arginine was required for the aimed-for Th1/Tc1-type immunity (IFN-gamma secreting T cells).

Keywords: Hepatitis C; Peptide vaccine; Randomized controlled trial; Human

1. Introduction

Hepatitis C virus (HCV) is responsible for the majority of both parenterally transmitted and community acquired non-A, non-B hepatitis. An estimated 170 million humans, or 1–3% of the world population, are infected with HCV [1] with an even higher prevalence in the third world. About 10 Million Europeans, 3.9 million Americans and 2 million Japanese are infected with HCV, and 35,000 new infections occur in the US alone each year (WHO, Weekly Epidemiologic Report, No. 3, 2000, 75). Since the current gold standard of treatment with pegylated interferon and ribavirin is curative in only about half of HCV patients [2], there remains an important need for alternative therapies and effective vaccines. The high unmet medical need is underscored by the fact that each year 8000–10,000 deaths and 1000 liver transplantations are due to HCV in the US (NIH Consensus Development Conference Statement, Management of Hepatitis C, June 2002; CDC: Fact Sheet Hepatitis C, http://www.cdc.gov).

HCV is a positive-stranded enveloped RNA virus belonging to the family of flaviviridae. Its 10 kilobase genome contains a single open-reading frame giving rise to a polyprotein

☆ Presented in part at the American Association for the Study of Liver Disease (AASLD) meeting in Boston, MA, October 29–November 2, 2004, and at the 40th Annual Meeting of the European Association for the Study of the Liver (EASL) in Paris, April 13–17, 2005. The protocol and all amendments were approved by the Ethics Committee of the Medical University, Vienna. The study was carried out in compliance and in accordance with good clinical practice (GCP), and all relevant guidelines of the international conference for harmonization (ICH). Written informed consent was obtained from all participants prior to study entry at the Department of Clinical Pharmacology, Vienna.

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which is posttranslationally processed into structural (Core, E1, and E2/NS1) and non-structural proteins (NS2, NS3, NS4, and NS5) [3,4].

Infection leads to viral persistence and chronic disease in ~80% of the cases [5], and sequels contribute significantly to morbidity and mortality. Chronic HCV infection over years leads to liver fibrosis and cirrhosis. Moreover, it is a leading cause of hepatocellular carcinoma (HCC) and liver transplantation, which often occurs despite humoral and cellular immune responses against the structural proteins of the virus [6,7].

Primary HCV infection causes broad and multispecific $CD4^+$ and $CD8^+$ T cell responses. It has been reported that stronger, broader and more sustained Th1/Tc1 (IFN-gamma) responses are associated with resolving infection [7–18]. Indeed T cell responses can readily be detected in humans in the absence of viremia many years after clearing infection [14,19–23]. Although chronically infected patients also show some IFN-gamma responses, these tend to be weaker and directed against less epitopes [21,23]. In addition HCV specific T cells appear impaired in chronic infection [15,24,25]. The high mutation rate of an RNA virus and the existence of quasispecies in the same individual facilitate immune escape mechanisms that can undermine productive T cell responses [16,26–33]. Additional potential immune deviations in chronic HCV include dysfunction of dendritic cells [34–38] and suppressor T cells [39–44].

The role of specific antibodies against HCV is more controversial. Envelope antibodies would be the prime candidates for virus neutralization, but their presence in chronically infected patients as well as in animal experiments argue against efficient humoral virus neutralization in vivo [45,46]. Further, the existing antibodies are mostly specific against HVR1 of the envelope protein 2. This is disadvantageous, because the heterogeneity in the envelope HVR [47] may be accompanied by the failure of the immune system to mount an antibody response to the dominant strain [48] and also to respond to interferon therapy. Finally, antibody-mediated immune pressure seems to directly correlate with an evolution of viral escape mutants during the course of infection [49].

In light of the above we hypothesized that eliciting an anti-HCV immune response based on the induction of epitope-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ responses may be highly beneficial. We also reason that it is advantageous to concentrate on well-conserved proteins using well-characterized epitopes spread over a large percentage of the population e.g. HLA A2-restricted epitopes.

This is reflected in the design of IC41 that contains at least four HLA-A2 restricted CTL epitopes and three highly promiscuous $CD4^+$ Helper T cell epitopes, all of which have been shown to be targeted in patients responding to standard treatment or spontaneously recovering from HCV (own unpublished data). The synthetic HCV peptides in IC41 are adjuvanted with poly-L-arginine, which has been shown to augment Th1/Tc1 (IFN-gamma) responses in animal studies [50–54].

Here, IC41 was investigated in a randomized, placebo controlled trial enrolling 128 HLA A2 positive healthy volunteers. This study was designed to examine the immunogenicity of different doses of IC41 with or without poly-L-arginine as adjuvant. An immunization schedule of four s.c. vaccinations, each 4 weeks apart was chosen on the basis of a previous phase 1 trial (own unpublished data). Here we report on the clinical outcome of this study (safety, overall immunological responses to vaccination), a detailed immunological analysis will be published elsewhere.

2. Methods

2.1. Vaccines

The IC41 HCV vaccine (Intercell AG, Vienna, Austria) consists of defined components: peptide antigens and poly-L-arginine, both synthesized by chemical means to high purity and consistency. In order to minimize viral escape, a pool of five different peptides (Ipep 83, 84, 87, 89, 1426) conserved in the most prevalent HCV genotypes 1a (100%, 100%, 83%,100%, 100% for the respective five peptides), 1b (98%, 90%, 15%, 94%, 88%) and 2 (91%, 96%, 13%, 91%, 87%) was employed. As IC41 harbours besides highly promiscuous T-helper epitopes, HLA-A2 restricted CTL epitopes, only individuals positive for HLA-A2 were enrolled in the study. The prevalence of this marker is 45–50% within Caucasians [55]. For the current study, several doses of IC41, HCV peptide vaccine only, poly-L-arginine only and saline were applied (see Table 1).

2.2. Study design and interventions

This was a single (subject) blind, randomized, controlled parallel group study for dose optimization and to assess safety of a HCV peptide vaccine in healthy subjects. Twelve

Table 1
Description of treatment groups

5 HCV peptides (mg)	Poly-L-arginine (mg)	No. of subjects
Control groups		
0	0	20
0	2.00	12
5.00	0	12
Treatment groups		
0.50	0.25	12
0.50	0.50	12
2.50	0.25	12
2.50	1.25	12
2.50	2.00	12
5.00	0.50	12
5.00	2.00	12
Total number of subjects		128

subjects were randomized into each group, except for the saline group which consisted of 20 subjects. Subjects received four vaccinations subcutaneously (one every 4 weeks for three months on Days 1, 29, 57 and 85). Blood samples for immunological assays and safety laboratory were taken at baseline, before each vaccination, and 4 and 12 weeks after the last vaccination.

Subjects eligible for inclusion were healthy male and female volunteers aged from 18 to 50 years positive for HLA-A2 (assays performed at the Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna). In addition, all subjects had to fulfill all inclusion criteria as follows: mentally and physically healthy, no clinically relevant pathological findings in any of the investigations of the pre-study examination (including blood chemistry, differential blood counts, coagulation test, ultrasensitive C-reactive protein levels). Exclusion criteria were as follows: positive results in the human immunodeficiency virus (HIV), hepatitis B surface antigen (HBsAg) or anti-HCV screening, history of autoimmune disease, donation of blood or blood components one month prior to enrolment, until study end, history of severe hypersensitivity reactions, anaphylaxis or atopy (excluding asymptomatic hay fever), known gastrointestinal, hepatic, renal, respiratory, cardiovascular, hematological, coagulation, metabolic or hormonal diseases, diseases of the central nervous system (such as epilepsy), psychiatric disorder or impaired cerebral function, malignancies, abnormal thyroid function indicated by abnormal thyroid-stimulating hormone (TSH) levels (substitution permitted), known history of orthostatic hypotension or fainting spells, active or passive vaccination within two months prior to enrolment, and concomitant vaccination throughout the study period, administration of any drug that could have influenced the immunological response within 4 weeks prior to enrolment, pregnancy or lactation, participation in another study with an investigational drug within one month prior to enrolment, excessive drinking habits (more than approximately 60 g alcohol per day), drug addiction; positive results in the urine drug screening, or a body mass index (BMI) $\geq 30\,kg/m^2$).

2.3. Safety assessments

Physical examination and vital sign checks were performed at every study visit. Safety monitoring was carried out at the study site by the clinical investigator before injection, 10 min and 1, 2 and 3 h after vaccination. In addition, the participants were requested to record any adverse reaction that might have occurred during the first 24 h post-injection on an individual diary card. To monitor acute safety, body temperature and vital signs were checked, and local (pain, itching, induration, edema, erythema), as well as systemic reactions were registered. The solicited adverse events (AE) were reported and analyzed separately from the non-solicited AE. Differential blood counts, urinalysis and blood chemistry were done at monthly or bimonthly intervals.

2.4. Outcome measures and statistical considerations

To assess vaccine immunogenicity, interferon-gamma ELIspot assay, T cell Proliferation assay, HLA tetramer-binding assay were carried out with cryo-preserved PBMC. These assays allow reliable measurements of epitope-specific T cell responses induced by the therapeutic HCV vaccine IC41. The vaccine-induced T cell immune responses serve as surrogate parameters of efficacy. [56] All immunological assays were validated and carried out conforming to Good Laboratory Practice (GLP). The study protocol defined the proliferation assay as the primary outcome variable. With HCV peptide specific T cell responses negative at baseline, any response at any timepoint during or after vaccination against any of the HCV peptides was regarded to be a vaccine induced response. Accordingly, proliferation class II (any of the three class II T helper epitopes of IC41), ELIspot class I (any of the four class I = HLA-A2 epitopes of IC41), ELIspot vaccine (any of the five peptides of IC41), FACS class I (any of the three class I = HLA-A2 epitopes tested) responders were defined. In addition overall vaccine responders were defined as any of the above. Responder definitions were purposely set as loose as possible to avoid underestimation of responder rates due to false-negatives, as direct ex vivo assays without in vitro pre-stimulation, and using cryopreserved PBMC may give very low yet meaningful antigen-specific T cell responses. The number of patients per group ($n = 12$) was an empiric number typical of early drug development, and was based on the results seen in a small first in man trial (own unpublished data). To allow a more precise estimate for the assays' specificity the sample size of the placebo group was larger ($n = 20$). No power calculations were performed and only descriptive statistics were applied for this exploratory trial. Complete HLA class I and class II typing was performed at the Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna and besides ITT analysis, immunological results were also analyzed based on each subjects HLA class I (HLA-A*0201) and class II (HLA-DR) background. As both analyses yielded comparable results, here consistently ITT analysis is presented. Data monitoring, and data management was performed by ICON Clinical Research GmbH (Germany).

2.5. Peripheral blood mononuclear cells (PBMC) preparation

Blood anticoagulated with acid citrate dextrose (1:9) was processed within 1 h after sample collection. PBMC were isolated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) using Leuco-sep tubes (Greiner, Frickenhausen, Germany), washed 3× with PBS (Invitrogen Life Technologies, Carlsbad, CA, USA) and resuspended at a concentration of $2 \times 10^7\,ml^{-1}$ in freezing medium consisting of nine parts foetal bovine serum (FCS; from PAA, Linz, Austria) and one part DMSO (SIGMA, Deisenhofen, Germany). PBMC were

stored over night in 1 °C freezing containers (Nalgene Nunc International, Rochester, New York, USA) at −80 °C and then transferred into liquid nitrogen.

2.6. Proliferation assay

The T cell proliferation assay was used to analyze HCV peptide-specific T helper cell responses, individually for each of the three peptides containing HLA class II epitopes. Cryo-preserved PBMC were thawed quickly in a 37 °C water bath, washed 1× with assay medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 μM 2-mercaptoethanol, 1% antibiotic/antimycotic (all from Invitrogen Life Technologies) and 5% human serum type AB (PAA, Linz, Austria) and incubated overnight (37 °C, 5% CO_2). The next day cells were plated at 50,000 PBMC/well (96-well plates) and co-cultivated in four replicates with individual peptides (10 μg/ml) for 6 days. After adding 1 μCi of [^{3}H]thymidine (Hartmann Analytic, Vienna, Austria) to each well cells were cultured for further 16–18 h. Then cells were harvested on Multiscreen Harvest plates (Milipore) and incorporated radioactivity was measured using a liquid scintillation beta-counter (MicroBeta Jet, Wallac, Perkin-Elmer). An HBV-derived HLA class II peptide was used as negative control. Concanavalin A was used as non-specific positive control. Tetanus toxoid (Statens Serum Institut, Kopenhagen, Denmark) was used as antigen-specific positive control. On each assay plate a PBMC sample with known reactivity against tetanus toxoid and the HBV negative-control peptide was developed as quality control. Stimulation index (SI) was calculated by dividing the median stimulated culture counts by the median of the negative-control (HBV-peptide) culture counts. A positive stimulation index was considered when SI was ≥4.

2.7. ELIspot assay

IFN-gamma ELIspot [57] was done individually for each of the five peptides of IC41, in addition, 2 HLA-A2 epitopes contained within longer peptides were tested individually. Briefly, Multi Screen 96-well filtration plates MAIP S4510 (Millipore, Bedford, MA) were coated with 10 μg/ml (0.75 μg/well) anti-human IFN-γ monoclonal antibody (Mab) B140 (Bender Med Systems, Vienna, Austria) over night at 4 °C. Plates were washed two times with PBS (Invitrogen Life Technologies) and blocked with ELIspot medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 μM 2-mercaptoethanol (all from Invitrogen Life Technologies) and 10% human serum type AB (PAA, Linz, Austria). Cryo-preserved PBMC were thawed quickly in a 37 °C water bath, washed 1× with ELIspot medium and incubated overnight (37 °C, 5% CO_2). The next day cells were plated at 200,000 PBMC/well and co-cultivated in six replicates with individual peptides (10 μg/ml) for 16–20 h. After removing cells and washing six times with wash buffer (PBS; 0.1% Tween 20 from SIGMA), 100 μl of a 1:10000 dilution (0.015 μg/well) of the biotinylated anti-human IFN-γ MAb B308-BT2 (Bender Med Systems), was added for an incubation of 2 h at 37 °C or alternatively for over night at 4 °C. After washing, Streptavidin-alkaline phosphatase (DAKO, Glostrup, Denmark) was added at 1.2 μg/ml for 1 h at 37 °C. The assay was developed by addition of 100 μl/well BCIP/NBT alkaline phosphatase substrate (SIGMA). Peptides from HIV (HLA-A2 restricted) and HBV (promiscuous T helper epitope) or medium without peptide served as negative controls. HLA-A2 restricted peptides from CMV, EBV and influenza served as specific positive controls, Concanavalin A as non-specific positive control. On each assay plate a PBMC sample with known reactivity against the CMV positive-control peptide and the HIV negative-control peptide was developed as quality control. Results were considered positive when the median spot number was at least three times greater than the median spot number obtained in the negative-control wells (HIV peptide; 0–5 spots per million PBMC in most assays) and at least 15 spots per million PBMC.

2.8. HLA-A*0201 tetramer-binding assay (FACS)

A five-color HLA-A*0201 tetramer-binding assay was used to quantitate HCV-specific $CD8^+$ T cells in PBMC. Tetramers for three HLA-A2 epitopes of IC41 were available. Briefly, cryo-preserved PBMC were thawed quickly in a 37 °C water bath, washed twice, and resuspended in PBS/0.5% bovine serum albumin/0.01% sodium azide. 1×10^6 cells were stained with the following monoclonal antibodies (mAb)/tetramers: anti-CD8-PC7 mAb (Beckman Coulter, Florida, USA), anti-CD4/CD13/CD19-PC5 mAb (i-MascTM Gating Kit; Beckman Coulter Immunomics, Marseille, France), anti-CD45RA-ECD mAb (Beckman Coulter Immunotech, Marseille, France), anti-CCR7-FITC mAb (R&D Systems, Minneapolis, USA), and HLA-A*0201-PE tetramers loaded with HCV, CMV, and HIV epitopes, respectively (Beckman Coulter Immunomics). After incubation at room temperature in the dark for 30 min, cells were washed twice with PBS/0.5% bovine serum albumin/0.01% sodium azide, fixed in 0.5% paraformaldehyde, and analyzed on a Cytomics^{TM} FC 500 flow cytometer (Beckman Coulter). For the analysis cells were gated on CD4-, CD13-, and CD19-negative cells first, then gated on a lymphocyte gate and finally on CD8-positive cells. Results were expressed as percentages of tetramer-binding cells in the CD8 positive population regardless of cytokine secretion pattern. Tetramer background staining was measured using an HIV tetramer, the CMV tetramer served as positive control. Intra-assay coefficient of variation of this assay was generally <4%. The limit of detection of the assay was calculated to be 0.04%, when the background staining of the negative control tetramer was set to 0.00–0.01% for each individual sample. A total of 50,000 events were acquired

in each analysis. In each assay PBMC with known reactivity against the CMV positive-control peptide and the HIV negative-control peptide was developed as quality control.

2.9. Poly-L-arginine antibodies

Analysis was conducted by a qualitative ELISA method (by Gene Logic Laboratories, MD 20879, USA) in compliance with the Food and Drug Administration Good Laboratory Practice Regulations as set forth in Title 21 of the U.S. Code of Federal Regulations Part 58. The method set-up at Intercell AG utilized poly-L-arginine and rabbit antiserum to poly-L-arginine, in conjunction with commercially available horseradish peroxidase (HRP) conjugated anti-human IgG (Sigma–Aldrich, St. Louis, MO), HRP conjugated anti-rabbit IgG (Intercell), and ortho-phenylenediamine (OPD; Sigma) HRP substrate in a 96 well microplate format. The rabbit antiserum served as a surrogate positive control utilizing the HRP conjugated anti-rabbit IgG. The detection was colorimetric using the static end point of OPD color development. Naïve human unique serum samples served as negative controls. A cut-off value was established for each assay run based on the naïve sera response ($n = 6$) in order to determine whether the samples are positive or negative for the presence of anti-poly-L-arginine antibodies. The cut-off values were calculated as three times the standard deviation (S.D.) of the mean response of the naïve unique samples (mean + 3S.D.). There were a total of four assays performed in support of this study. The assay cut-off values (positive/negative threshold) were established from the mean values of six individual serum samples + 3S.D. for each assay. Positive control samples prepared at the 1:100, 1:500, and 1:1000 levels were greater than the established cut-off values for each assay run. The positive control samples displayed precision as %CV results that were $\leq 15\%$ and the mean OD492 values for the positive controls decreased as the dilution increased.

3. Results

3.1. Subject characteristics

A total of 149 subjects were enrolled into the study, of which 128 were randomized and received study treatment, demographic data are presented in Table 2.

3.2. Safety results and adverse events (AE)

In general, the peptide vaccine was well tolerated. Local reactions were the most frequent adverse events that were considered *possibly or probably related to treatment.* Local intolerance was seen in half of the subjects treated (pain, edema, pruritus or induration each occurred in 8–17% of subjects) in the peptide only group, poly-L-arginine only group or active treatment groups, whereas no injection site reaction was seen after s.c. injection of saline. Intolerance to study vaccinations was mostly experienced at the time, or within 1 h of vaccination but persisted to a greater extent in the IC41 groups than in the control groups during the 24-h post-vaccination period. Other possibly *vaccination-related* AE included lymphadenopathy ($n = 2$) or exanthema ($n = 2$), arthralgia, dizziness, eczema, fatigue or pyrexia, each observed in individual subjects.

When considering *all* AE independent of relationship to study drug, headache was the most frequent AE and was observed in approximately one-fourth of all subjects treated, irrespective of group assignment. The second most common

Table 2
Demographic characteristics at baseline

	IC41 groups ($N = 84$)	Control groups ($n = 44$)	Total ($n = 128$)
Age (years)			
N	84	44	128
Mean ± S.D. (range)	28 ± 8 (18–49)	29 ± 8 (19–49)	28 ± 8 (18–49)
Sex, *n* (%)			
Male	38 (45)	19 (43)	57 (44.5)
Female	46 (55)	25 (57)	71 (55.5)
Race (%)			
Caucasian	99	98	98
Oriental	0	2	1
Other	1	0	1
Weight (kg)			
N	84	44	128
Mean (S.D.)	68 (13; (48–105))	68 (12; (48–107))	68 (13; (48–107))
Median	65	66	65
Weight group (%)			
<50 kg	1	4.5	2
50 to 90 kg	95	91	94
>90 kg	4	4.5	4

Table 3
Responder rates for T cell assays stratified by dose group

	Peptide (mg)/adjuvant (mg) (ratio)				
	2.5/0.25 (10:1)	2.5/1.25 (2:1)	2.5/2 (5:4)	5/0.5 (10:1)	5/2 (2.5:1)
High dose IC41 groups					
T cell proliferation (%)	75	67	75	67	50
$CD4^+$ cells ELIspot (%)	25	42	42	33	50
$CD8^+$ cells ELIspot (%)	0	25	0	17	42
FACS tetramer (%)	50	42	33	42	42
	0/0	0/2	0.5/0.25 (2:1)	0.5/0.5 (1:1)	5/0
Control groups and low dose IC41 groups					
T cell proliferation (%)	15	25	25	42	58
$CD4^+$ cells ELIspot (%)	10	0	17	8	8
$CD8^+$ cells ELIspot (%)	5	0	0	8	0
FACS tetramer (%)	0	0	25	25	33

adverse events were flu or flu-like syndromes which were encountered in 5–9% of study subjects, once again without noticeable differences between study groups.

Two serious adverse events occurred during the treatment period or within 30 days after the last vaccination: one was an injury of a palmar artery and nerve of the left hand after an accident, the other one was a planned abortion (her decision was not influenced by the fact that the women was in this study). These serious adverse events were judged not to be related to the study drug.

Two adverse events led to the withdrawal of subjects from the study: one was arthritic pain in the left forefoot in a young woman, with a family history of arthritis. Serologic workup revealed no evidence for serologic markers of a rheumatoid disorder or autoimmune disease. As we could not rule out that unspecific stimulation of T cell activity could exacerbate the disease process, we decided to discontinue treatment, and the subject recovered spontaneously. The other adverse effect which led to withdrawal of a subject was an induration at the vaccination site, which was probably related to treatment, and which led the volunteer to withdraw consent. The two subjects who were withdrawn (receiving peptide/adjuvant: 0.5 mg/0.5 mg and 2.5 mg/1.25 mg) were included in the total number of AE as well as in the intention to treat analysis.

The vaccination had no consistent effect on any determined safety parameter. Six subjects in the IC41 groups tested positive for poly-L-arginine antibodies during the study (6% of the 96 poly-L-arginine exposed subjects); four of these were re-tested at the follow-up visit (the other two subjects were declared "lost to follow-up") and only two remained positive. Positive responses for poly-L-arginine antibodies increased with number of vaccinations, although follow-up retesting indicated that poly-L-arginine antibodies were transient in nature. Furthermore, no allergic or anaphylactic reactions were observed. None of these six subjects had any related AEs, and none were withdrawn from the study prematurely. There was no apparent inhibitory effect of poly-L-arginine antibodies on immunological responses.

3.3. HCV peptide specific T cell proliferation

50 to 75% of volunteers who received 2.5–5.0 mg peptides ± poly-L-arginine showed T cell proliferation responses, as opposed to 25–34% in those subjects receiving 0.5 mg IC41 (Table 3). However, a false positive proliferation responder rate of 15% of subjects receiving saline only indicates that the specificity of the proliferation test was only about 85% using our responder definitions. "Sustained responses", defined as SI ≥4 against the same peptide in two consecutive visits, or three times within the five measurements during/after vaccination, occurred in three-fourth of the responders; importantly none of the three false-positives of the saline group was a sustained responder. Fig. 1 depicts the time course of HCV peptide specific T cell proliferation in response to IC41 vaccination in a sub-analysis pooling data

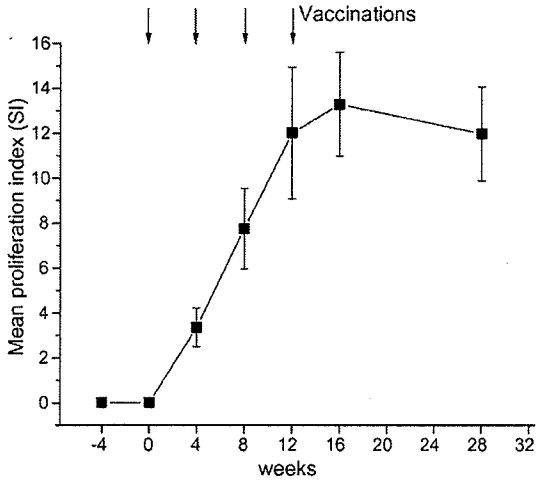

Fig. 1. Evolution of T cell responses over time as measured among 61 responders in T-cell proliferation assays (arrows indicate the times of vaccination). Stimulation indices for each of the three peptides tested individually were summed up for each responder, when above the threshold SI > 4. Data are presented as mean ± S.E.M. of all responders per time point.

Table 4
Correlation of proliferation, ELIspot and HLA-tetramer responses within individuals

	Subject	Proliferation $CD4^+$	ELIspot $CD4^+$	ELIspot $CD8^+$	FACS $CD8^+$
Peptide (mg)/adjuvant (mg) 5.0/2.0	3	×	×	×	×
	8				×
	32	×	×	×	×
	44	×	×		×
	49	×	×	×	
	86	×	×	×	
	95		×	×	×
	104	×			
Peptide (mg)/adjuvant (mg) 5.0/0.0	13		×		×
	24	×			×
	51	×			
	53	×			
	66	×			×
	87	×			×
	123	×			
	141	×			

Positive responder status is shown as "×". Groups with highest peptide concentration with or without adjuvant are shown as example.

from all 61 proliferation responders. This approach is more informative than analysis per group, which is compromised by the relatively high number of non-responders. HCV peptide specific T cell proliferation increased with the number of vaccinations and peaked after the last vaccination with a mean SI of about 13. As control, tetanus toxoid specific T cell proliferation was assessed in all subjects: 88–95% showed responses to tetanus toxoid, and the median SI fluctuated between 28 and 33 over time.

3.4. HCV peptide specific $CD4^+$ T cell interferon gamma ELIspot responses

Fifty percent of subjects who received 5.0 mg peptides plus poly-L-arginine had $CD4^+$ ELIspot responses, as compared to only one subject (8%) receiving 5 mg peptides without poly-L-arginine (Table 3). This indicates the necessity to include poly-L-arginine for inducing effective IFN gamma secretion by $CD4^+$ T cells by the peptides (Fig. 2). Twenty five to forty two percent responders were observed among those volunteers receiving 2.5 mg IC41. When the IC41 was further lowered to 0.5 mg, responder rates declined to 8–25%. Only 2/20 subjects receiving saline responded, which indicates that the specificity of the ELIspot $CD4^+$ T cell test was about 90% in the current trial. In general functional IFN-γ secreting $CD4^+$ responses were less prevalent than $CD4^+$ responses assessed by proliferation (Table 3). For correlation of responses within individuals see Table 4.

3.5. HCV peptide specific $CD8^+$ T cell interferon gamma ELIspot responses

Over forty percent of volunteers receiving 5.0 mg IC41 plus 2 mg poly-L-arginine showed ELIspot responses, as compared none receiving 5 mg peptides without poly-L-arginine (Table 3). Hence, the ELIspot assay indicates that poly-L-arginine mounts the peptide induced IFN-γ secretion by $CD8^+$ T cells (Fig. 2). Null to 25% responders were observed among those volunteers receiving 2.5 mg IC41 plus poly-L-arginine, and decreasing the IC41 dose further to 0.5 mg yielded a maximum of 8% responders in the $CD8^+$ ELIspot assay. Only one of 20 subjects receiving saline

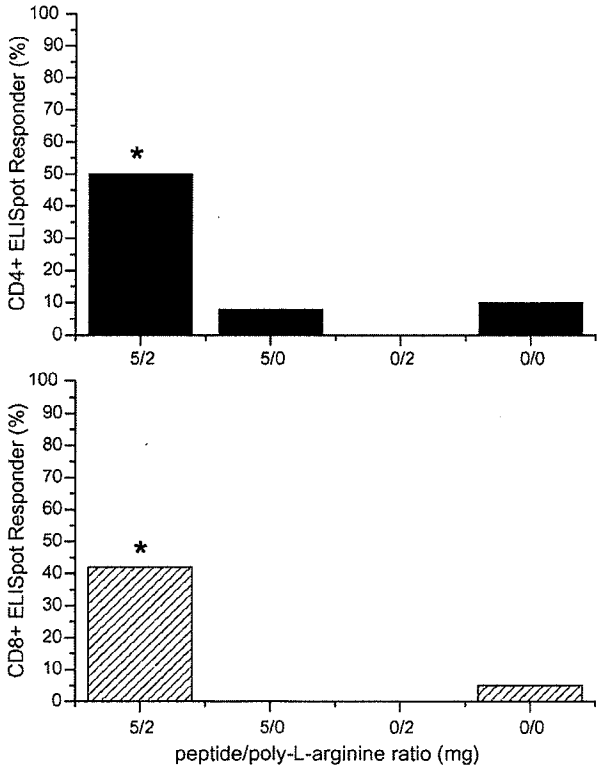

Fig. 2. Poly-L-arginine is a Th1/Tc1 adjuvant in humans. Percent responder rates as assessed by interferon-gamma ELIspot are shown for four different dose groups. *Top*: $CD4^+$ Helper T cell responses. *Bottom*: $CD8^+$ cytotoxic T cell responses. $^*p < 0.025$ vs. other groups by Chi squared-test.

Table 5
Total ELIspots elicited through IC41

Peptide (mg)/ adjuvant (mg)	Sum of vaccine (ELIspots per Mio PBMC)	
	Baseline	Maximum
0/0	0	15
0.5/0.25	0	15
0.5/0.25	0	15
2.5/0.25	0	15
2.5/1.25	0	15
2.5/2.0	0	15
5.0/0	0	15
5.0/0.5	0	15
5.0/0.5	0	15
5.0/2.0	0	15
0/0	0	20
2.5/2.0	0	20
2.5/1.25	0	25
2.5/0.25	0	30
5.0/2.0	0	30
5.0/2.0	0	30
2.5/2.0	0	35
5.0/0.5	0	35
5.0/2.0	0	40
5.0/2.0	0	40
2.5/0.25	0	45
2.5/2.0	0	45
5.0/0.5	0	45
0.5/0.5	0	70
2.5/1.25	0	70
2.5/1.25	0	85
5.0/2.0	0	85
2.5/2.0	0	120
2.5/1.25	0	185

Sum of vaccine is calculated by adding up ELIspots measured individually against each of the five peptides of IC41 after subtraction of background (irrelevant HIV peptide subtracted). The maximum sum of vaccine (usually recorded after three or four vaccinations) is shown in comparison to baseline (week 0, first vaccination).

responded, which indicates that the specificity of the $CD8^+$ ELIspot test is about 95% in the current trial.

The total ELIspots elicited through IC41 are shown in Table 5 for each individual responder. About 40% showed weak responses (15–20 spots per million PBMC), these included the two false-positive responders in the saline group and the single ELIspot responder in the 5 mg peptide only group. The other responders distributed equally between 25–35, 40–45, and 70–185 spots per million PBMC (each group ~20%). The median within all 29 ELIspot responders was 30 spots per million PBMC. As controls HLA-A2 peptide specific recall responses against CMV (36–38.5% responders), influenza (86–77% responders) and EBV (82–84.5% responders) were recorded in all assays. The median EBV specific ELIspot counts fluctuated between 63 and 85 per million PBMC over time showing the robustness of the assay.

3.6. HLA-A2 tetramer FACS assay

Forty-two to 50% of volunteers who received 5.0 or 2.5 mg IC41 showed HLA-A2 HCV epitope specific responses in the tetramer assay, as opposed to 25–34% in those subjects receiving 0.5 mg IC41 plus poly-L-arginine. Similarly 34% responders were seen in the 5 mg peptide only group. No response was seen in the poly-L-arginine- only group and 5% of subjects responded in the group receiving saline only. The latter group indicates that the specificity of the tetramer assay is about 95%. As control HLA-A2 peptide specific recall responses against CMV were recorded in all assays. In general the non-functional tetramer binding assay gave higher responder rates than IFN-γ secreting $CD8^+$ responses assessed by ELIspot (Table 3). For correlation of responses within individuals see Table 4.

4. Discussion

In this study, T cell immuno-reactivity in response to IC41 was assessed using [^{3}H]-thymidine proliferation, IFN-γ ELIspot assays and HLA tetramer flow cytometry (FACS). These assays enable measurements of epitope-specific T cell responses induced by vaccination with IC41. Cryo-preserved blood cells were used, to allow direct comparison of before/after treatment samples of individuals in one assay. This resulted in a possible underestimation of T cell responses compared with assays that utilize fresh blood. However, due to the lack of inter-laboratory standardization of T cell assays, direct comparison of the results of this study with published data from similar studies is not easily possible.

Our results summarized in Table 3 show that class II responder rates (T cell proliferation or $CD4^+$ ELIspot) were more frequent than class I responder rates (FACS or $CD8^+$ ELIspot). This is not unexpected, since it is a common finding that class I restricted $CD8^+$ T cells are more difficult to induce than $CD4^+$ T cells, partially due to the higher prevalence of the latter. For class II, T cell proliferation responder rates were greater than $CD4^+$ ELIspot vaccine responder rates. For class I, FACS responder rates were greater than $CD8^+$ ELIspot rates. Both results support the concept that generation of a functional IFN-γ secreting T cell response (i.e. those T cells quantified in the ELIspot assay) is more difficult than the generation of any T cell response (i.e. T cells secreting other or no cytokines at all). This is generally reflected in the responder status for the different assays within individuals as shown for two dose groups in Table 4.

Comparable T cell proliferation responder rates were observed in the high dose IC41 groups and the peptide only control group. In contrast, $CD4^+$ or $CD8^+$ ELIspot responder rates were greatest in the IC41 groups (Table 3, Fig. 2). This finding implies that co-administration of the adjuvant poly-L-arginine enhances the induction of functional IFN-γ secreting T cells in humans. Previous animal studies have already indicated that poly-L-arginine is an effective adjuvant [50–54]. Mechanistically, poly-L-arginine induces a depot-effect [54], enhances antigen uptake by HLA class II antigen presenting cells [50], in the skin, and enhances the expression of HLA

class II (HLA-DR) and co-stimulatory molecules on the surface of mononuclear cells (own unpublished data).

Antibodies to poly-L-arginine became detectable in 6% of volunteers receiving either peptide/poly-L-arginine or poly-L-arginine alone. There was no apparent correlation with the dose. Natural IgM antibodies cross-reactive with polycationic peptide stretches and hence also poly-L-arginine may exist in normal healthy subjects [58]. Of note, this occurrence of antibodies was not associated with exanthema. This is of interest because drug-induced systemic lupus erythematosus is associated with the production of antinuclear antibodies. These autoantibodies react with histones, which are rich in arginine residues. Symptoms of this disease usually resolve within several days to weeks after the responsible medication is discontinued.

One of the objectives of this study was to determine the optimal dose for immunization with IC41. A summary of IC41-induced T cell responses to the different doses is presented in Table 3. There was a peptide dose dependent response as assessed by proliferation ($CD4^+$ T cells) and FACS ($CD8^+$ T cells), with 5 mg and 2.5 mg groups virtually indistinguishable. In these cytokine secretion independent assays there was no apparent adjuvant effect. On the other hand, responder rates assessed by IFN-γ ELIspot were dependent on the adjuvant particularly for $CD8^+$ cell. The highest responder rates were recorded with the 5 mg peptide together with the 2 mg poly-L-arginine, which supports the choice of this dose combination in further trials.

Although several reviews have been published on the potential development of vaccines against hepatitis C [46,59–61] original reports on the immunogenicity of hepatitis C vaccines in humans are limited to one specific vaccine. An open-label, single-armed trial was conducted in 19 subjects who received a C-terminally truncated form of the envelop protein E1 (20 μg formulated on aluminium hydroxide in a 0.5-ml dose). Three vaccine doses were injected i.m. at 3-week intervals and a fourth (booster) dose was given at week 26 (±4 weeks) in a study extension protocol. Seroconversion occurred in all subjects, and T cell proliferation occurred in approximately 90–100% of subjects after three and four vaccinations [62]. Due to the lack of a placebo group, it is however difficult to draw conclusions on the specificity of the assay. In a subsequent placebo-controlled phase II trial in 35 patients, a T cell proliferation response was observed in 68% of patients after the first course of five vaccinations, and in 88% of patients after the second course of six vaccinations [63]. In comparison to response rates to the envelope protein E1, and also with other peptide-based vaccines mainly applied in the field of cancer immunotherapy [64], responses to IC41 peptides appeared to occur with almost similar frequency. However, since only less than half of the vaccinees developed $CD8^+$ and $CD4^+$ IFN-γ responses, future trials aiming at optimization of IC41 immunogenicity are required.

In conclusion, the current study is unique in that it tested a novel concept, i.e. the vaccination with short synthetic hepatitis C virus peptides in humans, which may be of conceptional interest for a variety of diseases including cancer. Immunization with IC41 was generally safe and well tolerated. In addition, poly-L-arginine increased the number of subjects who mounted an IFN-γ secreting T cell response, providing *proof of concept* for this adjuvant in humans. It has to be noted that any HCV-peptide specific response measured at any time-point during or after therapy against any of the vaccine peptides in any of the three assays applied (proliferation, ELIspot, HLA-tetramer) was recorded as vaccine response. This certainly does not correspond to a productive immune response in HCV infection. However, it has not been established which quality and quantity of an immune response is required to achieve clinical responses in HCV patients. These tantalizing questions will only be answered through controlled clinical studies in HCV patients.

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